"LUMINESCENCE OF NUCLEIC ACIDS AND NUCLEIC ACID-BOUND DYES"

by

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PREFACE

This is the second semi-annual report to be issued on grant no. NGR 26-001-018, entitled: "Luminescence of Nucleic Acids and Nucleic Acid-Bound Dyes", for a grant period of one year beginning September 1, 1973. The major effort during this period was expended towards a review of the literature in the field, identifying promising avenues of investigation and prospective applications of new technology, and initiating an untried course of experimental investigation. During this period all of these aims have been met.

The work described in this report is the sole effort of the principle investigator, applying 10% of his time above his academic commitments during the academic year, September 1973 to May 1974, and 100% during two and one-half months, June 1974 to August 1974. The former represents 25% of the project research time available, during which the literature survey and part of its evaluation was completed; during the latter period further evaluation and research decisions and preparations were made and experimental investigation of novel acridine dyes was undertaken.

This report consists of two parts. Part A is a summary of the literature survey, the products of which is still undergoing sifting and study. Part B describes the theme, the direction and the early results of the experimental investigation.

Notice has been received of continuation grant support for another year, which, appropriately, will allow for proper exploration and development of nucleic acid-selective tailored acridines, which should serve as macromolecular conformation probes.

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TECHNICAL REPORT

Part A. Literature Survey

I. SUMMARY

The literature survey has collected pertinent articles of studies of dye--nucleic acid interactions. Several classes of dyes are found to undergo profound binding with nucleic acids (c.f. II-D). Of these, ethidium bromide and acridine orange represent the best fluorescence indicators for evaluating the presence of nucleic acids at low concentrations. The fluorescence of ethidium bromide undergoes a 25-fold increase in intensity upon binding to nucleic acids.

Several acridines, such as proflavin, show a diminution of fluorescence upon binding. These dyes, however, will stain the nuclei of biological cells in vivo, and would be very valuable if the binding effect on fluorescence could be overcome. A remarkably high detection sensitivity is claimed for the binding of cellular nucleic acids by acridine orange. This latter dye has been much studied.

Acriflavin and ethidium bromide are commercially employed as trypanocides. Other drugs, such as tetracyclines, also deserve future study as fluorescence probes of dye-nucleic acid interactants, since literature reports of fluorometry of various nucleus-binding drugs is sparce and skimpy.

Improvement in the present state of affairs appears to lie in the area, aside from the inspection of those new nucleus-binding drugs which fluoresce, of the synthesis of new acridine and ethidium bromide analogs. A chemical structural feature among most of the dyes which bind nucleic acids

strongly is present in 3,6-diaminoacridines, which contributes both to good fluorescence and to effective strong nuclear binding. Appropriate substitution, based on the 3,6-diaminoacridine structural core, seems to hold the most immediate promise of new dyes which would maximize the desired binding and luminescence properties. Part B of this report is based on an extension of this conclusion.

II. LITERATURE DIGEST

A thorough literature search was undertaken to compile and digest a bibliography of reported studies of luminescence of nucleic acids and nucleic acid-bound dyes. The literature search was conducted in three phases, as follows:

Phase I. Search of Chemical Abstracts from 1946 to the present, including recent unbound, non-indexed issues, for all apparently relevant citations, represents the main entre to the literature. Other ancillary sources have also been consulted. A searcher's record was kept of citations.

Phase II. All citations of Phase I were checked through abstracts or, when in doubt, by reference to the original papers. The abstracts of pertinent papers were recorded for future purposes.

Phase III. Original papers have been collected for experimental details of value for analysis, tabulation or summary.

Phase I, completed for nucleic acids, employed the keywords: luminescence, fluorescence, fluorimetry, phosphorescence, scintillation, nucleic acids-deoxyribo, nucleic acids-ribo, nucleosides, nucleotides, books, Phase II resulted in the selection of 256 abstracts. The completion of Phase II allowed grouping into subjects sufficient for the formation of decisions on the experimental program. However, analysis of details of each paper for a substantive grasp of the field is a slower process, which will continue into the experimental phase, over the course of the entire grant period.

II. SUMMARY

The major goal in reviewing the literature on the luminescence of nucleic acids is to identify systems which will lend themselves to an analytical determination of these biopolymers. The desirable features of an analytical procedure are that it be simple and applicable to automatic instrumental operation, that it be highly sensitive, and that it be specific. The literature is being canvassed for the best known methods, and for clues to methods worthy of further examination and development. The literature search indicates that one of the necessary criteria, i.e. high sensitivity, in a fluorometric assay is achievable by presently known methods.

A. Luminescence of Biopolymers in the Cell.

The first investigation showed that cells of microbial, plant, and animal origin exhibit intense ultraviolet luminescence on excitation at 250—280 nm. (1). The protein content of nucleoprotein and related cellular materials is mainly responsible for absorbed quanta of this wavelength range, and present evidence indicated that the origin of ultraviolet fluorescence is protein, and in particular largely due to tryptophan. Although the removal of free nucleotides RNA, and DNA from the cell has been shown to be connected with a sharp reduction of the luminescence, nucleic acids at room temperature and physiological pH show negligible native luminescence, especially when properly purified. It is known that nucleic acid components--guanine and adenine--fluoresce in highly acidic media, and energy transfer produces phosphorescence and thermoluminescence emission attributable to the presence of thymidine. Such native luminescence has been reviewed in detail. (2). It has been established that cell luminescence is tied to the functional state of the cell. Thus, cells of different tissues differ

in their luminescence; luminescence is altered by a change in the functional physiological state of the cell, and conversion of the cell to the pathological state is accompanied by corresponding changes in luminescence.

The desirable goal of high sensitivity in a luminometric assay method for nucleic acids will be met more easily by the involvement of dyes that interact with nucleic acids, rather than by the native luminescence of cells.

B. Fluorescent conjugates of Nucleic Acids.

Nucleic acids have been tagged by reaction with a fluorescent dye. The procedure is that of Zamecnik, et al and others (3), by converting the ribose of the terminal nucleoside to a dialdehyde by treatment with periodate, and then condensing with the amino group of a dye, such as acriflavin. However, this process introduces only one acriflavin label into a polynucleotide molecule, and thus places limits on the achievable maximum fluorescence intensity, although the method has general applicability and is important for monitoring intramolecular transitions and molecular interactions of polynucleotides, since many parameters of fluorescence, e.g. polarization and quantum yield, are influenced by such changes.

C. Indirect methods.

Histones, protamines, phosphatases and other substances are often closely involved with cellular nucleic acids. Methods can be evolved, based on such substances and many clinical assay methods exist of this kind (4). The use of a fluorogen, e.g. riboflavin phosphate, provides a degree of freedom with regard to the question of fluorescence intensity, since a phosphatase enzyme may convert many molecules of the fluorogen, thus, multiplying its presence many times over. However, from the point of specificity, such methods suffer from being indirect, adding a measure of interpretation and running the risk of false positive indications. Further, the enzyme would be present in considerably lower concentration than the nucleic acid itself. More promising is the exploitation of the strong direct binding of dyes to nucleic acids.

D. Direct dye-binding to nucleic acids.

Many basic dyes bind so strongly to nucleic acids that they are mutagenic. Of these dyes some show a good facility of permeating cell membranes which permits in-vivo nuclear staining. The general procedures for staining live cells with fluorescent dyes has been reviewed (5). Binding of dyes to nucleic acids takes place by more than one process; there is strong binding commonly considered to occur by intercalation, and there is weak binding by electrostatic attraction of the dye molecules to the outside of the polymer. In strong binding, up to one dye molecule per 4—5 nucleotides may be found bound; in external binding, as many cationic dye molecules will bind as there are negative phosphate groups to the point of electric neutrality. There is also dye-to-dye aggregation which can increase the amount of dye binding in the latter situation, but this may be accompanied by metachromatic changes in the dye absorption and luminescence spectra, often occuring with a loss in fluorescence efficiency. At higher concentrations cell components other than the nucleus take up the penetrating dyes.

Among the well known dyes, which have been used for many years for vital staining in microscopy are methylene blue (MB), acridine orange (AO), eosin (ES), neutral red (NR) pyronine (PN), toluidine blue (TB), as well as colored antibiotics (e.g. actinomycin) and such dyes as are natural components of the cell or very similar to these components (e.g. riboflavin or lumichrome). Of interest are the fluorescence studies which locate the binding of various tetracyclines to mitochondria (6) and various macromolecules (7), for reconsideration as the basis of an assay method. More recently introduced dyes are thiopyronine (TP), furocoumarins, and ethidium bromide (EB).

Such strongly bound dyes can produce photodynamic action, which if thoroughly characterized may serve for specific confirmation. Strong binding has been demonstrated in single-stranded nucleic acids. Strong binding of

fluorescene dyes to nucleic acids can cause decrease in fluorescence efficiency, as occurs with proflavin. Several covalent closed cyclic DNA's are in vitro at least superhelical, and also show strong binding. Strongly bound acridine to DNA dissociates only when DNA looses its secondary structure upon heating at high temperature; native DNA above 40°C actually has a higher binding capacity than denatured DNA. Changes of ionic composition, and of concentration, as well as temperature, change the conformation of nucleic acids, the extent of binding, and therefore, fluorescence efficiencies. Ionic strength, pH, detergents and other components of the external medium can affect the degree of cell penetration by a dye. Obviously, many parameters must be evaluated before proposed dye systems are considered as a prime candidate for an assay method.

The chemical literature on nucleic acid-dye fluorescent systems shows the following pattern. The volume of publications in this area has undergone a marked increase since 1967, and several books and reviews which in part cover the subject have been published between 1969 and 1972. (8). Review of the literature from 1947 to the present shows that major effort recurrently focusses on a few systems, AO, and other acriflavins; EB (since its introduction); the Roberts-Friedkin method of bromination of thymine-containing nucleotides, followed by the condensation of acetol and o-aminobenzaldehyde to give 3-hydroxyquinaldine; the Kissane-Robbins method of Velluz in which deoxyribose mers are reacted with 3,5-diaminobenzoic acid (a very sensitive method: 0.002 μg DNA), Auromine O, and modifications of the Feulgen reaction (a Schiff reagent of basic fuchsin and sulfurous acid) to give highly fluorescent products, as with Acridine-Y-SO_2 and Acriflavin-SO_2. Publications reporting refinements of these methods appear every year.

Acridine orange applications receive continuing attention. Much of the information and theory concerning the interaction of AO and nucleic acids and

detailed analysis of the spectra have been reviewed (9). The lifetime of the AO-DNA complexes could be used to detect double-stranded and single-stranded zones in the nucleic acid (1o), which is also indicated by changes in the absorption and fluorescence spectra. DNA irradiation damage is also detectable by such color changes (11). Combined fluorescence staining with AO and 3,4-benzpyrene serves to identify DNA (green), RNA (orange red), and lipids (yellow) in the same cell(12). A remarkabley high sensitivity is claimed in detecting 8×10^{-14}g. of the nucleic acid, i.e. that corresponding to a single bacterium (13). These results should be independently confirmed.

Since its introduction ethidium bromide, a trypanocide, has received much ~~acids~~ attention (14). Unlike most acridines, (but not all) EB shows upon binding to nucleic acids 25-fold increase in general fluorescence intensity and up to a 100-fold increase when measured at its maximum emission wavelength 590 nm. The hyper fluorescence is influenced by the conformation of the polymer, since EB binds to poly(vinyl sulfate) without showing an increase in fluorescence. It remains to be shown whether other rigid rod polymers can produce a similar effect with EB as nucleic acids do. The configurational state of various nucleic acids has been examined with EB (15). Improved DNA methods have been since reported with the use of EB (16). Microdetermination of nucleic acids (17), DNA in human granulocytes (18), DNA and RNA in tissue homogenates (19), DNA in bacteria (20), DNA interstrand crosslinks (21), and bihelicity (22) have seen most recent applications with EB fluorometry.

Binding of AO and of EB to nucleic acids show specific effects in relation to structural conformations of nucleic acids. In addition, the use of nucleases before and after fluorometry, lends additional confirmation of the presence and chemical constitution of the nucleic acid substrate. These disciplines can be exploited to build certainty into the interpretation of fluorimetric results.

Both of the above methods (AO + EB) are claimed to give very high sensitivity. If further development for these or other dyes are shown not to be limited by sensitivity requirements, additional specificity can be introduced into any method at the expense of some loss of emission intensity by using polarizers in the optical system.. Since their use reduces background scatter as well, the signal-to-noise need not suffer the same magnitude of loss and the sensitivity of the method can be regained by electrical amplification. The use of polarizers is of interest because dyes, which show no fluorescence polarization in the unbound state in solution, show a high degree of polarization when bound to DNA by intercalation (23). The engineering requirement is to orient the stained-DNA sample, as by flow through a capillary.

Acridine dyes have been investigated for fluorescence polarization (23). Some acridine dyes are non-fluorescent in aqueous solution and fluoresce when bound or when localized in viscous media (24). Acridine dyes show a good facility to permeate cells and intercalate into DNA. This faculty correlates with the basicity of the acridine dyes. Fluorescence, however, in many cases is decreased by binding because of Forster type energy transfer. Knowing the molecular parameters which govern these processes makes attractive the prospect of synthesizing new acridine dyes which would maximize the best properties. Nucleic acid-acridine complexes, including proflavin and AO, have been investigated to gain insight into mechanisms of energy transfer (25), which, while detracting from the ability of acridine to fluoresce, contributes to their ability to phosphoresce, or to undergo photochemical reactions (24). Fluorescent staining of DNA by atebrine has been used to distinguish human spermatozoa bearing X, Y or YY chromosomes (26). On the whole, though, the number of papers devoted to the use of acridine dyes, with the notable exception of AO, have decreased markedly.

The Roberts-Friedkin DNA assay (27), the Kissane-Robins Ribose assay (28),

and the Feulgen (29) reactions, (and modifications thereof) receive continued usage. These methods, however, introduce greater complexities with regard to automated remote-control assays because of the manipulative steps involved.

Other agents reported recently which have been used in luminescence studies are Auramine-O, and Auramine-OO (30), malonaldehyde (31), terbium ions, which complex and fluoresce (32), and silver ions (33), coriphosphin (34), and berberin sulfate (35).

E. Oligonucleotides

Fluorometric and phosphorimetric techniques for the determination of purines, pyrimidines, nucleosides, nucleotides, coenzymes and derivative thereof are also receiving heavy coverage (36), as they have for years (37). Purines will form complexes with hydrocarbons (38). Some of these methods may become applicable to nucleic acids, such as has the Roberts-Friedkin and other examples described above. A recent significant advance in this area is the synthesis of intensely fluorescent derivatives by reaction of nucleic acid constituents with chloroacetaldehyde (39) or with glyoxal hydrate trimer (40).

F. Instrumentation

The sensitivity of an analytical method depends in part on instrumentation. For instance, low levels of emission intensities depend on collection and discrimination from background noise. Improvements have been made is instrumental technology, especially developed for some other forms of spectrometry, such as with C^{13} nuclear magnetic resonance, employing repeptitive pulsing, multichannel excitation and detection and Fourier transform. Rapid electronic spectral scanning of millisecond periods is achieved with commercially available oscilloscopes employing silicon vidicon detectors. An experimental apparatus designed to minimize scattered light and based upon photon counting, shows forty fold higher sensitivity, linear fluorometry with concentrations of aqueous riboflavin

solutions as low as 4×10^{-11} g/ml (41).

Rather interesting is the recent application of a relatively new fluorescence methodology, correlation spectroscopy (42), which has been applied to DNA and EB fluorescent complex formation and to rhodamine 6G diffusion (43). The method is a departure from conventional fluorescence spectroscopy and incorporates several characteristics which, on the face of it, represent significant improvements in sensitivity and specificity.

DNA and RNA determinations by the fluorescence of the EB-complex has been automated (44).

Part B. Experimental Report

I. SUMMARY

Focus on the desirable fluorescence and in vivo binding properties of acridine orange, proflavin and euflavin led to consideration of overcoming the problem of fluorescence quenching by nucleic acids that occurs upon binding of these dyes. From this arose the concept of synthesizing binuclear acridines. At least one dye of the appropriate prescribed structure, bis-trypaflavin, was known to exist to this investigator and has been procured. Correspondence with Dr. Adrien Albert, acknowledged authority on acridine compounds, provided some additional acridines but revealed that most other appropriately structured binuclear acridines will need to be synthesized.

Bis-trypaflavin does not fluoresce in aqueous solution. However, conditions have been identified whereby the presence of nucleic acids induces a weak fluorescence. Thus, like ethidium bromide, this dye shows increased fluorescence upon interaction with nucleic acid. Furthermore, instead of a twenty five-fold increase in fluorescence intensity, bis-trypaflavin shows the analytical advantage of an intensity increase from some value close to zero. The induced fluorescence of bis-trypaflavin is affected by pH, by dye concentration, by the absolute and relative concentration of nucleic acids, and by

the viscosity of the medium. It is probably also affected by the temperature of the system and the nature of the nucleic acid. These parameters remain to be investigated in the forthcoming year, partly for the purpose of maximizing the fluorescence output, but, more interestingly, also for the purpose of understanding the possibilities of use of the dye as a macromolecular conformational probe of nucleic acids.

Further improvement in the development of the theme involves modification of the structure of bis-trypaflavin, focusing on the position and the length of the chemical bridge between the halves of the binuclear acridine.

II. THEME OF NUCLEIC ACID-INDUCED FLUORESCENCE

Acriflavin and its components, euflavin and proflavin, are known to bind nucleic acids very strongly by an intercalation process--in vivo and in vitro. Acridine Orange, a closely related acridine dye, and other dyes do likewise. One detracting property in the use of acriflavin is the quenching of fluorescence that occurs due to the interaction of the intercalated dye with the purine-pyrimide base pairs of DNA between which the dye is sandwiched. This proposal has as its thesis a route of investigation which will allow the strong intercalation phenomenon to take place, but will attempt to avoid fluorescence quenching by use of biacridinium dyes, i.e. more generally designed, by the use of dyes of A-B type, wherein A represents a strong intercalating moiety and B represents a pendant dye of good fluorescent properties, and A may equal B or be different.

Of the various dyes which bind to nucleic acids, several have the common structural feature of a cationic central binding site and two lateral amino groups which are separated by a distance approximating the crossection diameter of double stranded DNA, ca. 11 Å (see Figures 1 - 3). These include methylene blue, thiopyronine, ethidium bromide, as well as Auramine O and the

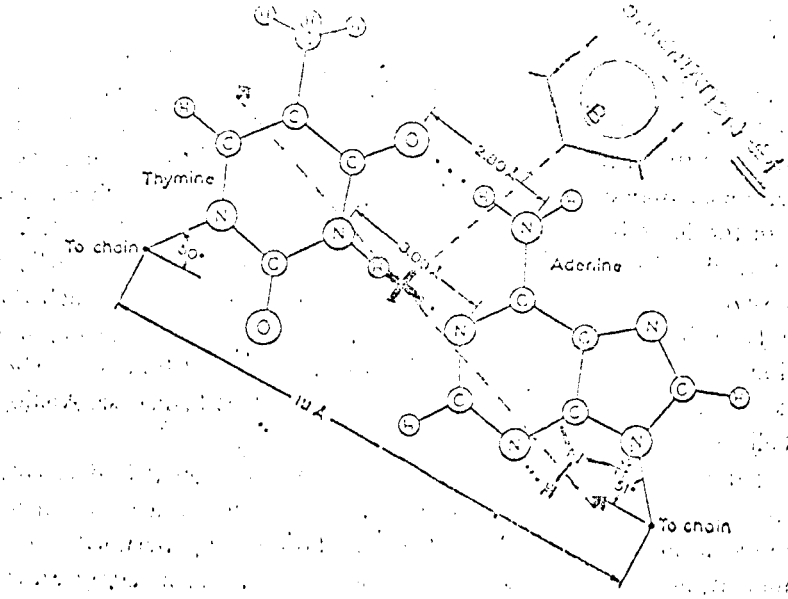
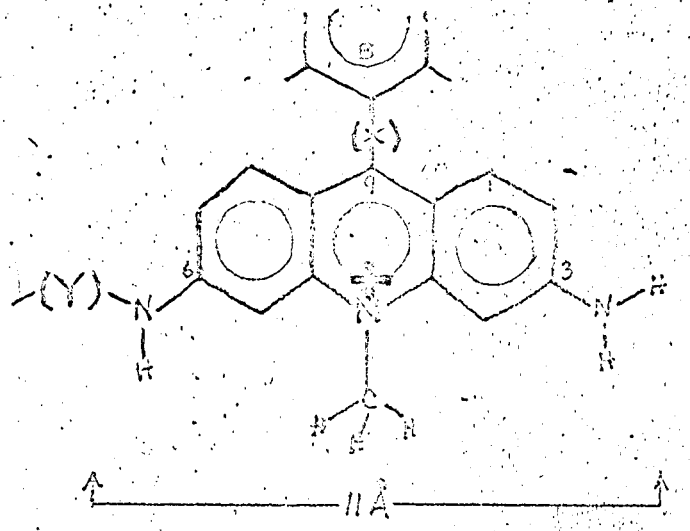
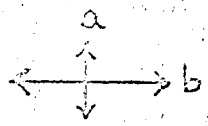


Fig. 1. Adenine-thymine pairing in DNA.





Major resonance vectors in acriflavin.

Fig. 3. Biacridinium dyes(3,6-diamino): where X = nothing, O, -CH=, or $NHCH_2CH_2NH$, and Y = H

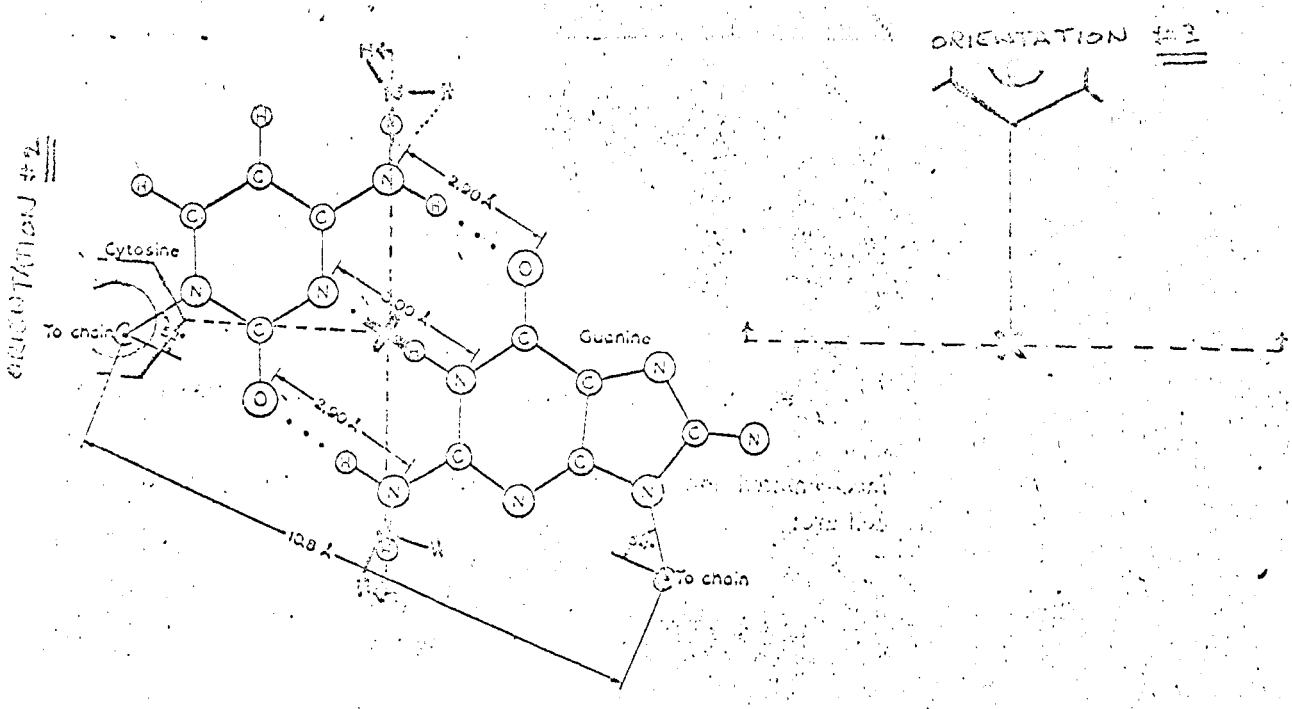


Fig. 2. Guanine-cytosine pairing in DNA.

acridines mentioned above. The positions of these three nitrogen atoms appears critical to strong nucleic acid binding. Figures 1 and 2 show two purine-pyrimidine base pairs, and Figure 3 shows one half of a biacridinium dye, drawn to scale. The dotted lines and cross that are drawn over Figures 1 and 2 illustrate three of several orientations that a 3,6-diaminoacridine may assume, but differ in rotation about the center cross. The cross represents the point at which the quaternary nitrogen of the dye may replace one proton that is normally delocalized between a base pair of nitrogen atoms. Biacridines present the additional consideration of spacial accomodation of the remaining bulk of the dye molecule.

In orientation 1, Figure 1, it can be seen that the pendant half finds room in the region of the deep grove of the DNA helix, probably with some tilting out of plane. (It should be remembered that the bridge between the two halves of the dye may be lengthened by appropriate synthesis.)

In orientation 2, Figure 2, it can be seen that the pendant half is in steric conflict with the atoms of the polymer backbond. Hence, this orientation is excluded to biacridines, but not the monoacridine dyes with which we are familiar.

The three nitrogen atoms of acriflavin lie on a straight line. Consequently, the pendant half may emerge into the free region of the DNA groove at one of two sides of the helix.

The intercalation process is pictured as a slight expansion of the helix with an insertion of the dye between layers formed by the base pairs. This brings the aromatic π-bonds into close overlap, known as "stacking". The pendant groups, if juxtaposed would be no closer than twice the distance between dye and base pair. And, if found alternately on opposite sides of the helix, would be no closer than four times this distance. As the dye/nucleic acid ratio of concentrations is kept low, the pendant dye halves would be even

further separated. It is well known that aggregating dyes show metachromatic absorption bands that are indicative of dimers and higher aggregates, but when isolated on a polymeric substrate can show the monomeric properties of absorption and high fluorescence. Such expected fluorescence in acriflavins are distinguished by spectral and intensity shifts. This proposal intends to examine the fluorescence of the pendant groups at various dye/nucleic acid ratios, for biacridines joined at C(9)——C(9'), and for the yet unknown dimeric dyes joined by a bridge attached to the 3-amino group: structures with (X) vs. those with (Y), resp. (c.f. Figure 3).

This project should further illucidate the orientation of the dye moiety in the base pair sandwich, from its spectral properties. Resonance in acriflavin has two major orthogonal vectors (see inset, Figure 3). A change in orientation, by rotation about the helical axis must affect the spectral shifts observed upon intercalation. Let us assume that orientation 2 (Figure 2) is prefered for monomeric dye, i.e. acriflavin, but that the corresponding biacridine can only intercalate through an orientation orthogonal to this, e.g. orientation 3. The spectrum of bound dye should show distinctive differences.

While orientation 2 is precluded sterically for a 9-9'-biacridine, it is a feasible orientation for N-bridged, 3-aminosubstituted biacridine, i.e. "Y"-type, Figure 3. Hence, the interest in coupling two euflavin molecules at the 3-amino groups with α,ω-dibromoalkanes of various alkyl lengths. These compounds have never been made before. They may have physiological drug action. Thus, a spin-off benefit may be derived from these syntheses. It might be expected that such binuclear binding dyes may act to physically crosslink otherwise separate helices.

The "X"-type biacridines are known. Prof. Millich has already obtained 50 mg. of "bis-trypaflavin" from his past research of acridine dyes. Bis-trypaflavin is the 9,9'-dimer of euflavin. It was found by Prof. Millich to be non-

fluorescent in water, but strongly fluorescent when in a very viscous medium, such as frozen glycerin. It is expected to show a similar viscosity dependent fluorescence when bound to and localized on nucleic acid. Fluorescence enhancement would serve as a very sensitive analytical tool, since the fluorescence would rise from zero intensity. Experimental work has just begun with this dye.

Prof. Millich also has in his possession N,N'-bis(9-acridyl)ethylene-diamine, which is moderately fluorescent. This compound lacks the 3,6-diamino groups, and thus would lack a fixed orientation should it intercalate, and might provide an interesting contrast for spectral comparisons.

Prof. Millich has corresponded with Prof. Emeritus Adrien Albert of the Research School of Chemistry, The Australian National University, Canberra, A.C.T., the acknowledged expert in acridine synthesis for several decades. Prof. Albert has forwarded a sample of 1,2-diacridylhydrazine, and offers samples of luzigenin and some other biacridines of the 9,9'-type. 9,9'-Biacridines have been reported in which X is an ether oxygen atom (flexible), and in which X is a methinyl -CH= group, such as is found among cyanine dyes (inflexible). If the pendant group is deep in the helical groove (short bridge), and there is a requirement of tilting to fit the pitch of the groove, this cyanine type compound, which links A- and -B halves of the dimeric dye by resonance, may not be able to maintain planarity. A more extensive cyanine-type bridge, (CH=CH)-CH=, can avoid this problem. It would be interesting to know how resonance between halves A and B respond to the effects of binding one half of the molecule into the fluorescence quenching intercalation.

This project may be summarized by saying that dye molecules of A-B type will be bound to nucleic acids, in which moiety B will serve as a flag that hopefully will be sensitive to conformation of nucleic acids, to rotational orientation of the dye around the helical axis, to delineating the spectral

changes for the two major resonance vectors of acriflavin, and to the pitch of the deep groove of the DNA helix. Such results would undoubtedly vary for different nucleic acids and for single and triply stranded polynucleotides. Finally, a spinoff benefit from the drug screening of any new biacridines is a possibility.

III. EXPERIMENTAL PROGRESS

The structures of bis-trypaflavin and proflavin are: related as a dimeric and monomeric form of the same structure; this relationship is valid at values of pH below 9, at which the nitrogen of proflavin in the 10-position is protonated in analogy with charged 10-methyl-nitrogen of bis-trypaflavin. Both dyes were submitted to parallel spectral investigation for purposes of comparison in expectation of some similarities.

Initial examination of these dyes was carried out at pH values 3—4. Proflavin exhibits intense fluorescence as a mono-cation; its pK_a constants at room temperature are reported as 9.7 (central nitrogen atom at position 10) and ca. 1.5 (first of two amino groups nitrogen atoms at positions 3 and 6). De Bruyn reports that proflavin, when bound to nucleoprotein, shows maximum fluorescence around pH 4 (45).

Absorption spectra of both dyes were obtained on a Cary 14 scanning spectrophotometer at pH values -0.3, +2, 4 and 10, at the concentration level of 10^{-5} mol/l. Proflavin in aqueous solution at pH 4 shows no deviation from Beer's law with increasing concentration as regards wave length distribution or the molar absorbance index, over a range of concentrations from 2×10^{-6} to 6×10^{-5} mol/l (24). However, it does show self quenching of fluorescence over this range (24). Once conditions were found that elicited some faint fluorescence from bis-trypaflavin, it was observed that the wavelength distribution of the fluorescence is concentration dependent. Quantitative data of the concentration dependences of absorption and fluorescence at

selected ranges of pH will be determined, as an essential step in the thorough definition of the luminescence properties of these two dyes.

The point which should be made here is that, although the absorption spectrum of proflavin, by example, remains constant at one concentration level throughout the range of the mono-cation (ca. 2—9), the fluorescence shows no such restriction, because, unlike the ground state of the dye, the excited state will have its own properties. For instance, its largest pK_a may be as many as five orders of magnitude smaller than that of the ground state. Similarly, its tendency to self-aggregate may also differ. A thorough collection of spectral data is an essential prerequisite.

The absorption spectra of proflavin and bis-trypaflavin at pH 4 are shown in Figures 4-A and 4-B, resp. It is seen that the spectra are similar, but differ in that the spectrum of bis-trypaflavin shows maxima at longer wavelengths. Both dyes show absorptions in two spectral regions: the ultraviolet and the visible range; both these ranges may serve for excitation of the dyes.

The fluorescence of proflavin at pH 4 was examined on a specially constructed Perkin-Elmer scanning, single-beam spectrophotofluorimeter, Model 195 of the Slavin-Palumbo type (46), which automatically records corrected spectra. A schematic diagram is shown in Figure 5. The excitation spectrum of proflavin closely mimics the absorption spectrum of this dye; however, excitation of the visible absorption band (λ_{max}=445 nm) is considerably more effective in producing a measurable fluorescence. Both luminescence spectra are shown in Figure 4-A.

The fluorescence of bis-trypaflavin at pH 4 in aqueous solution was unobservable at 0.8×10^{-5} mol/l. However, in the presence of 3 g/l of nucleic acid, a weak fluorescence spectrum was observed. This fluorescence was used to record an excitation spectrum which corresponds to the visible absorption band of bis-trypaflavin: λ^{ex}_{max}=480 nm. Thus, the desired effect of

producing a nucleic acid-induced fluorescence has been observed. It remains to find the best conditions needed to maximize the effect, and to hopefully increase what presently appears as a weak fluorescence. The fluorescence of bis-trypaflavin is given in Figure 6-A. Note the peak at λ=513 nm. Additionally, fluorescence is observed with a band centered about λ=540 nm, although at the instrumental conditions (i.e. wide slit widths) that was necessitated by low fluorescence intensity, stray light effects will have to be eliminated to establish the center of this band with certainty.

The fluorescence of bis-trypaflavin is observable in frozen glycerin (24). Apparently, as the motion of the dye is diminished, the dye is less able to dissipate the energy of excitation by rotation and vibration modes or by collision with solvent, and is thus prone to emit this energy as photons of fluorescence frequencies.

The fluorescence of bis-trypaflavin was also determined in glycerin at room temperature (Fig. 6B), and a maximum is observed at λ_{max}=530 nm. The spectrum differs from that observed in the presence of nucleic acid; however, upon five-fold dilution, to the level of 10^{-6} mol/l, the spectrum changes toward resembling the nucleic acid-induced fluorescence spectrum.

One striking, characteristic distinction of the nucleic acid-induced spectrum at high dilution is the occurrence of a fluorescence band around 513 nm, the region of the peak of proflavin fluorescence. A tentative hypothesis which may explain these results assumes that the fluorescence observed in glycerin at extremely dilute solution is characteristic of non-aggregated bis-trypaflavin or of half of the molecule, and this spectrum is observable in nucleic acid-associated dye at high concentration levels because of the ability of nucleic acids to isolate single molecules of the dye in separated binding sites of the polyelectrolyte molecule.

It is thus seen, that at one dye concentration, i.e. 10^{-5} mol/l, the spectra for nucleic acid bound-bis-trypaflavin is distinctive.

Studies of the dependences of the fluorescence spectrum on the absolute nucleic acid concentration, on the nucleic acid/dye ratio, and on the viscosity of the medium (to eliminate the last parameter from consideration) are indicated.

Various polynucleotides--RNA, DNA, synthetic, single, double and triple-stranded, of varying helicity--and nucleoproteins need to be examined. Further, the length and the position of the atomic bridge between the two halves of the binuclear dye need to be varied, in order to fully explore this novel theme. Continuation of grant support for another year will allow the exploration of some of these features.

LEGENDS TO FIGURES 4 AND 6

Figure 4. A. Proflavin Spectra. Curves B, C and D are absorption spectra: B at pH 4.0, C at pH 7.0 and D at pH 10.0. Curve E is the excitation spectrum at pH 4; observed $\lambda_{fl.}$ = 505 nm. Curve F is the fluorescence spectrum at pH 4; $\lambda_{exc.}$ = 445 nm.

B. Bis-Trypaflavin Absorption Spectra. A at pH -0.3, B at pH 4.0, C at pH 7.0 and D at pH 10.0; all at equal concentrations.

Figure 6. Bis-Trypaflavin Luminescence Spectra.

A. In the presence of 3 g/l nucleic acid at pH 4.0; concn. 10^{-5} mol/l.

B. In glycerin, in the presence of pH 4-buffer.

6A-1. Fluorescence, $\lambda_{exc.}$ = 475 nm; 6A-2. Excitation, $\lambda_{fl.}$ = 513 nm; 6A-3. Fluorescence, $\lambda_{exc.}$ =475 nm. Curve H: concn. 10^{-5} mol/l; curve L: concn. 2 X 10^{-6} mol/l; 6A-4. Excitation, $\lambda_{fl.}$ = 530 nm.

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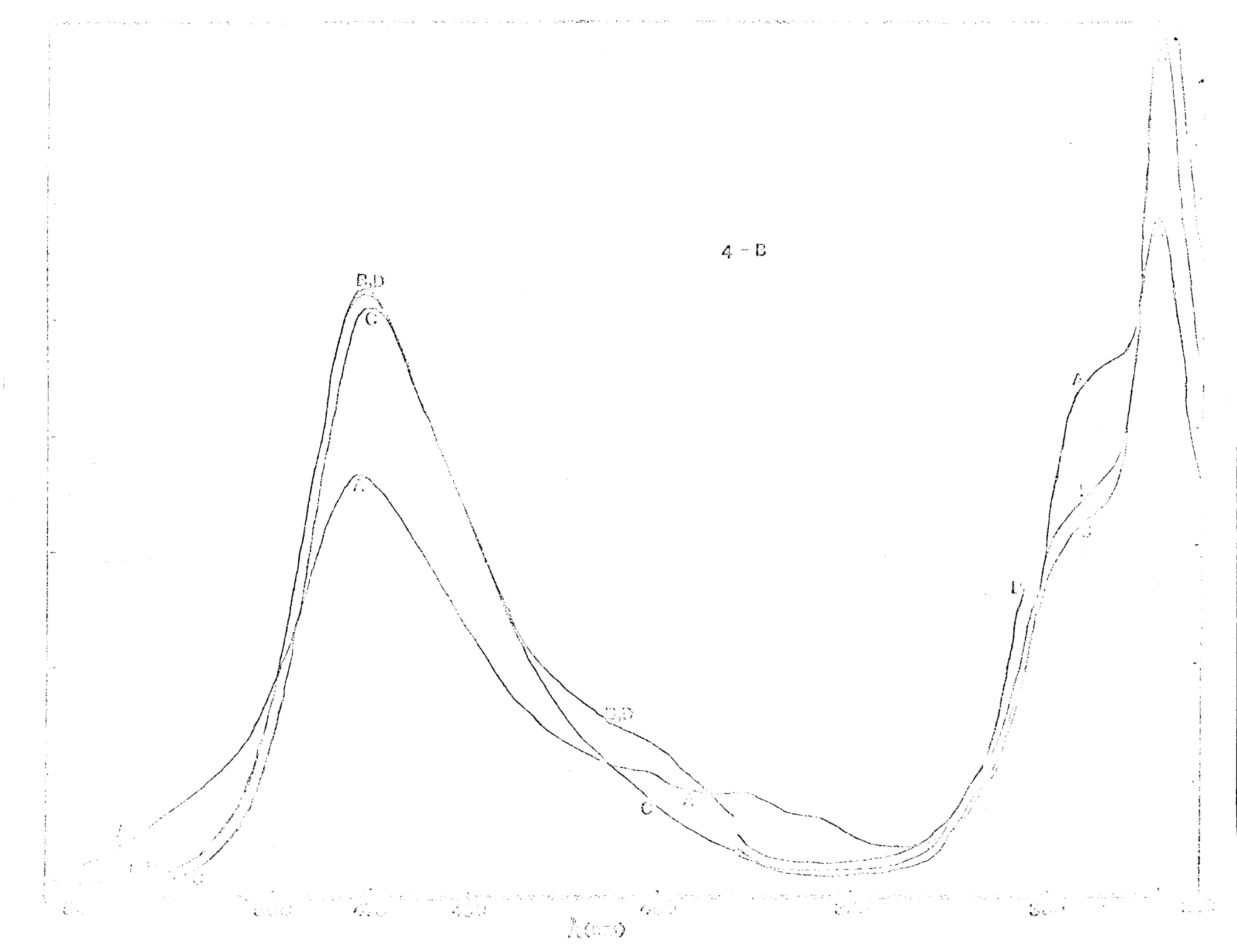
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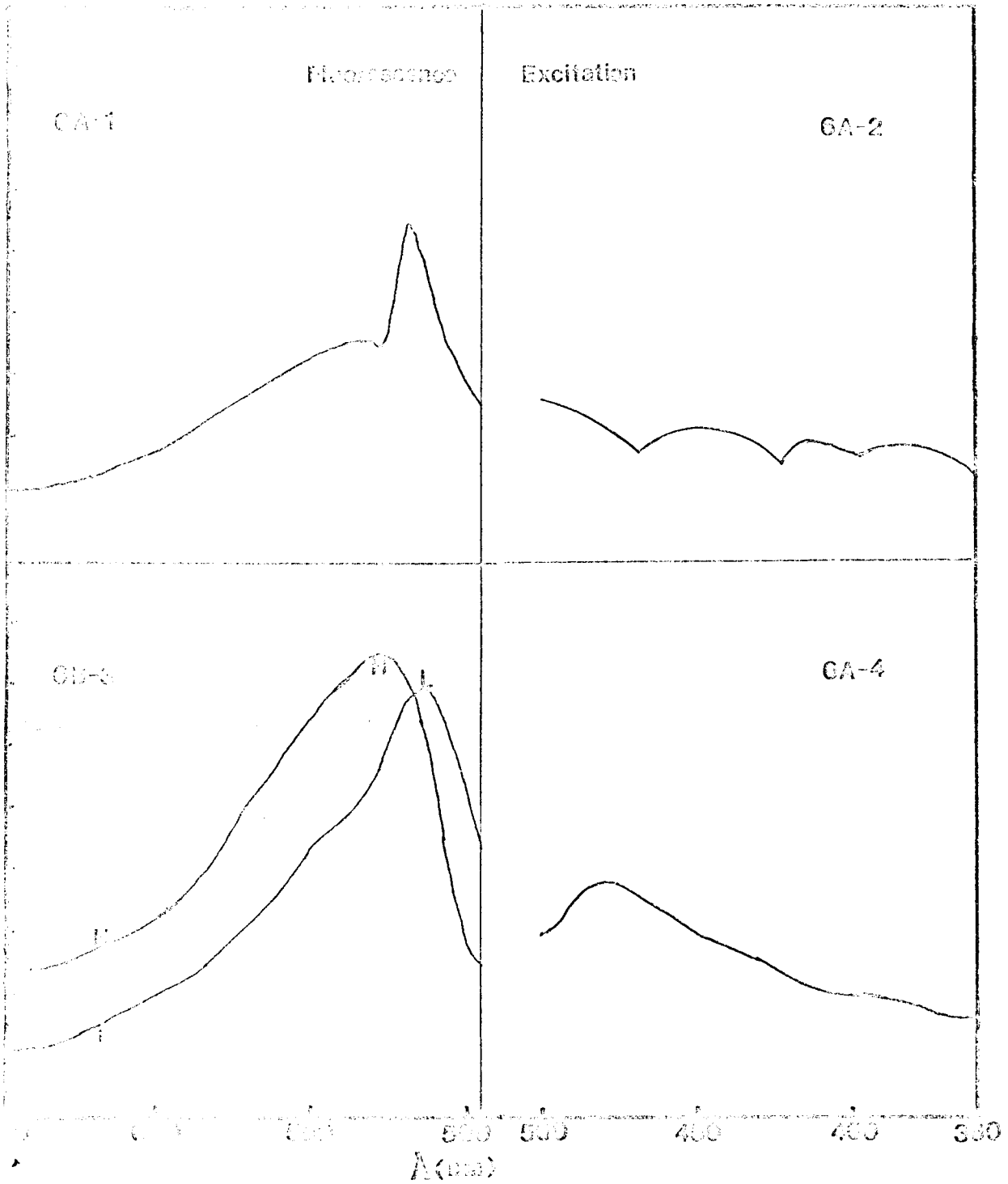

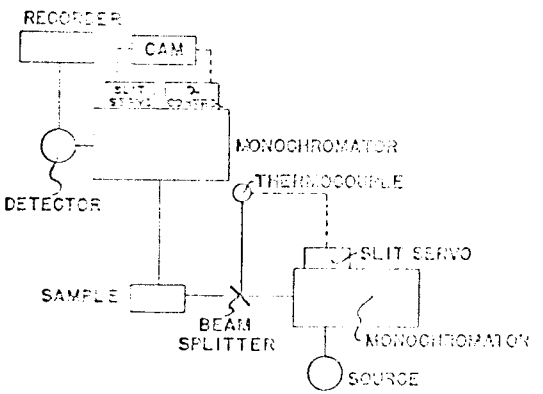

FIG. 5. System diagram showing the two servo control loops.